Demonstration of Skn Antigens on Mouse Epidermal Cells by Immunofluorescence and Flow Cytometry

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C57BL/6 (B6) mice that were lethally irradiated and reconstituted with (B6XA/J)F_1 spleen cells were immunized against the skin-specific antigens, Skn, by grafting with A/J tail skin. Serum from these mice was shown to contain Skn^a-specific antibody by a flow cytometric assay using indirect immunofluorescence. *J Invest Dermatol 88:574–576, 1987*

C57BL/6 (B6) mice that are lethally irradiated and restored with (B6XA/J)F_1 spleen cells [hereafter designated (B6XA)/B6 radiation chimeras] reject skin grafts from A/J (A) or (B6XA)F_1 donors [1]. Chimeras made by reconstituting inbred mice (B6) with hematopoietic cells of hybrid mice (B6XA)F_1 of a cross between the host strain and the prospective skin graft-donor inbred strain (A), are not affected by graft-vs-host disease since no element of the recipient is foreign. The skin graft rejections occur even though the mice are in good health with a normal life span and remain persistent hematopoietic chimeras [1]. Concomitant with graft rejection is the generation of antibody that reacts with A strain skin epidermal cells, but not with A strain hematopoeitic cells, as determined by the complement-dependent cytotoxicity assay [2]. This immunologic reaction is thought to be directed against the skin-specific antigens, Skn [3]. In this report, we confirm the presence of Skn-specific antibody in serum from (B6XA)/B6 radiation chimeras that reject A strain skin using indirect immunofluorescence and flow cytometry. This assay provides an objective and reliable system for the immunogenetic analysis of Skn antigens.

MATERIALS AND METHODS

Mice and Radiation Chimeras The C57BL/6 and A/J mice were obtained from the Jackson Laboratories, Bar Harbor, Maine. The (B6XA) mice were bred in the animal facility at the University of New Mexico. Only female mice were used in these experiments. The (B6XA)/B6 radiation chimeras were produced by irradiation of B6 mice with 1000 rad from an x-ray source at a dose of 75 rad/min. Within a few hours the mice were restored with an i.v. injection of 40×10^6 (B6XA)F_1 spleen cells in a volume of 0.2 ml of saline. The mice were treated with penicillin (90 U) and streptomycin (90 mcg) by i.p. injection every 2nd or 3rd day for 3 weeks and were allowed to recuperate for at least 6 weeks before grafting. Mice were randomly tested for chimerism using a complement-dependent cytotoxicity assay with anti-H-2^a and anti-H-2^b antibody.

Grafting and Antisera Grafts (1×1 cm) from tail skin were placed on a bed prepared on the dorsal thorax of (B6XA)/B6 radiation chimeras and secured with sutures. Serum for the assay (referred to as anti-Skn^a serum) was collected from mice 15–29 days after one graft with the following exceptions. In 1 mouse, serum was taken 8 days after rejection of a third A graft. In 4 mice, which had rejected a single A skin graft, serum was collected 6 days after receiving an i.p. injection of 2.5×10^6 A epidermal cells. Blood was collected from the tail vein; the serum was separated and stored at −70°C.

Preparation of Epidermal Cells Epidermal cells were prepared by the method of Scheid et al [2] with some modifications. The entire piece of skin covering the tail of adult mice was removed. The tail skin was incubated in Hanks' balanced salt solution (HBSS) without Ca^{++} and Mg^{+} containing 0.5% trypsin (1:250, Difco Laboratories, Detroit, Michigan) for 45–60 min at 37°C to separate the dermis from the epidermis. The dermis was discarded. The epidermis was placed in Medium 199 (M199, Gibco Laboratories, Santa Clara, California) containing 10% fetal calf serum (FCS) and 0.05% DNase (Sigma Chemical Company, St. Louis, Missouri). The medium was pipetted vigorously over the epidermis to free the epidermal cells. The resulting suspension was filtered through a 44-μm filter (Tetko, Inc., Elmsford, New York), washed in M199 with 5% FCS, and adjusted to a concentration of 5×10^6 cells per ml in HBSS plus 2% IPT (gamma globulin-free fetal calf serum, Gibco). In some of the later tests, after the initial trypsinization, the epidermal sheet was placed in 0.3% trypsin in HBSS without Ca^{++} and Mg^{+} plus 0.01% DNase for 5 min at 37°C. Medium 199 with 10% FCS was added and cells were disaggregated by agitation with minimal pipetting. They were then treated as already described.

Absorption of Antiserum Aliquots of antiserum diluted 1:8 in HBSS with 2% IPT were absorbed with epidermal cells of the required specificity by mixing 3 vol of antiserum to 1 vol of washed, packed epidermal cells. The suspensions were incubated

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Abbreviations:
A: A/J
B6: C57BL/6
FACS: fluorescence-activated cell sorter
FCS: fetal calf serum
FITC: fluorescein isothiocyanate
HBSS: Hanks' balanced salt solution

at 4°C for 30 min with gentle agitation. The absorbed antiserum was recovered by centrifugation.

Staining of Epidermal Cells and Analysis Using Indirect Immunofluorescence and Flow Cytometry Antiserum was diluted in HBSS with 2% IPT. All reactions were carried out at 4°C and washed in HBSS with 2% IPT. Fifty microliters of antiserum (1:8 dilution) or HBSS with 2% IPT (control) were added to an equal volume of prepared cells and incubated for 30 min. One of the procedures below was then followed: (a) Cells were washed 2 times followed by a 30-min incubation with 50 μl fluoresceinated antimouse IgG [fluorescein-conjugated sheep antimouse IgG F(ab')$_2$, Jackson Immunoresearch Laboratories, Inc., Avondale, Pennsylvania, or fluorescein isothiocyanate (FITC) goat antimouse IgG (H+L), Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Maryland]. (b) Cells were washed once followed by a 20-min incubation with 50 μl biotinylated horse antimouse IgG (Vector Laboratories, Inc., Burlingame, California), washed again, and then incubated for 10 min in FITC-Streptavidin (Amersham Corp., Arlington Heights, Illinois). Finally, the cells were washed twice and resuspended in 0.85% saline containing 1% paraformaldehyde. The cells were analyzed for fluorescent staining with a fluorescence-activated cell sorter (FACS III) flow cytometer (Becton, Dickinson and Co., Mountain View, California) equipped with a 488-nm argon ion laser (500 mW) and neutral density filter and interfaced with a Digital PDP 11/23 computer. Either 5,000 or 10,000 cells were analyzed for low, forward-angle light scatter and fluorescence. The LACEL (Los Alamos National Laboratories, Los Alamos, New Mexico) programs were used for data acquisition and analysis. The percent positive cells was determined by a modification of the method of Hoffman and Hansen [4]. The peak fluorescent channel of the control sample was identified and the number of cells in those channels below the peak channel was determined. The shift of cells out of these channels was quantitated after staining with anti-Skn^a serum and designated as percent positive cells.

Statistical Analysis A two-tailed Student's *t*-test was used to evaluate data.

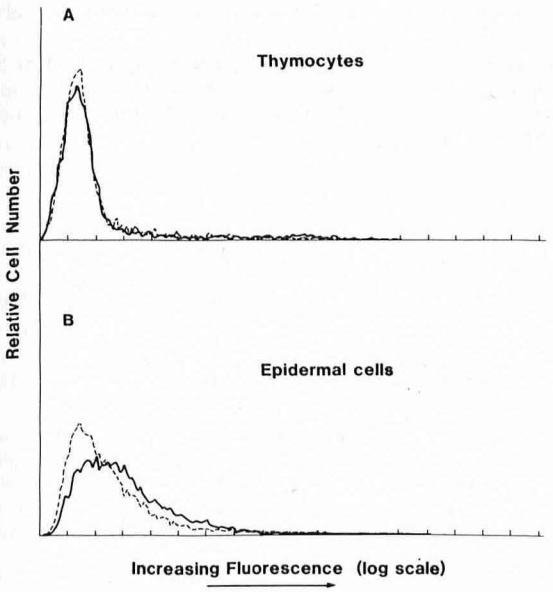

Figure 1. Fluorescence histograms of cells treated with anti-Skn^a serum. A strain thymocytes (*A*) and epidermal cells (*B*) were treated with anti-Skn^a serum (*solid line*) or medium alone (*broken line*) prior to staining with fluoresceinated antimouse Ig.

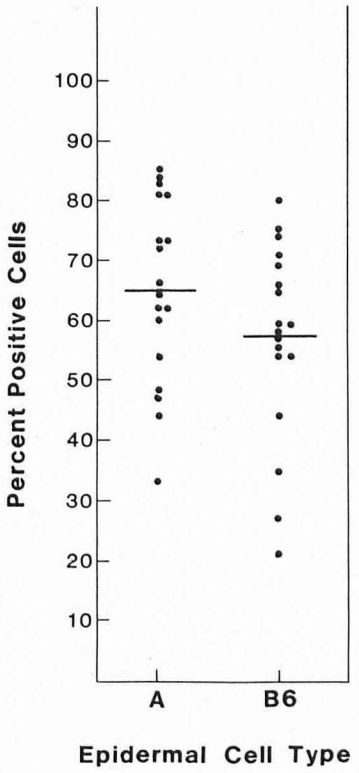

Figure 2. Percent positively stained A and B6 epidermal cells with anti-Skn^a serum. Anti-Skn^a serum was obtained from (B6XA)/B6 chimeras that have rejected an A strain skin graft. The mean (±SD) percent positive cells, indicated by the *horizontal bars*, is 65(±15) and 57(±16) for A and B6 cells, respectively.

RESULTS

The histograms illustrated in Fig 1 show that A strain epidermal cells are positively stained by anti-Skn^a serum (Fig 1*B*), whereas thymocytes (Fig 1*A*) treated in the same manner are negative, indicating antibody specificity for antigens present on normal skin epidermal cells. The shift in fluorescence obtained with anti-Skn^a-stained epidermal cells, although moderate in intensity, is highly reproducible.

To test for specificity of the reaction for A strain epidermal cells, anti-Skn^a serum was tested against A and B6 epidermal cells. As shown in Fig 2, although the percentage of positive cells was higher for A epidermal cells (65%) than for B6 epidermal cells (57%), the difference was not significant when analyzed statistically.

For this reason, we absorbed aliquots of the anti-Skn^a serum with either B6 (irrelevant) or A (relevant) epidermal cells prior to testing on A and B6 epidermal cells. The data shown in Fig 3*A* clearly indicate a difference in reactivity against A cells (40%) as compared with B6 cells (25%) when the serum was previously absorbed with epidermal cells of the B6 type. Despite the heterogeneity in the percentage of positive cells detected, statistical evaluation establishes a significant difference in staining of A vs B6 cells ($p < 0.01$). On the other hand, absorption of anti-Skn^a serum with A epidermal cells reduced the percent positive reaction against A to background levels (Fig 3*B*). These results indicate that although anti-Skn^a serum reacts with a certain percentage of B6 and A epidermal cells nonspecifically (much like we see when nonimmune "normal" mouse serum is used), this nonspecific reactivity can be removed by absorption with the irrelevant cell type.

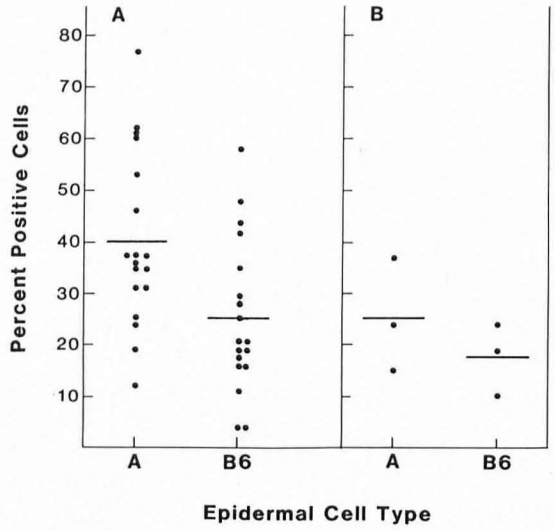

Figure 3. Percent positively stained A and B6 epidermal cells. *A*, Using anti-Skn^a serum that had been previously absorbed with B6 epidermal cells, the mean (±SD) percent positive cells, indicated by the *horizontal bars*, is 40(±17) and 25(±15) for A and B6, respectively ($p < 0.01$). *B*, Using anti-Skn^a serum that had been previously absorbed with A epidermal cells, the mean percent positive cells is 25(±11) and 18(±7) for A and B6, respectively ($p > 0.30$).

DISCUSSION

In the present study, using indirect immunofluorescence and flow cytometry, we have shown that (B6XA)/B6 hematopoietic chimeras that have rejected an A strain skin graft produce antibody specific for A strain epidermal cell antigens. Arguments supporting this conclusion are based on experiments showing that (1) the antigens recognized by anti-Skn^a serum are expressed on A strain epidermal cells but not on thymocytes; (2) the antiserum reacts with A, not B6, epidermal cells, indicating specificity of the reaction for Skn^a; (3) absorption of the serum with B6 epidermal cells enhances the difference in percent reactivity of A vs B6 cells such that the difference is significant; and (4) absorption with A epidermal cells removes the reaction against A cells to background levels. These findings are consistent with data previously reported using a complement-dependent cytotoxic reaction for the detection of Skn [2].

The nonspecific reactivity is partially removed by absorption with B6 epidermal cells. There have been a number of reports demonstrating the presence of autoantibodies to various skin components both in diseased and healthy tissue. We cannot rule out the possibility that the (B6XA)/B6 chimeras are producing autoantibody to B6 epidermal cell antigens. In mice, autoantibody to epidermal cells has been found [5] but evidence for the existence of a specific Skn autoantibody has not yet been demonstrated.

That heterogeneous cell types are present in epidermal cell preparations was obvious from the broad curve seen with the light scatter histograms and has also been shown by light microscopy of these preparations [6,7]. It is probable that Skn is expressed on only certain cell types in the epidermis and the density per cell most likely varies. There have been recent reports demonstrating the use of flow cytometry for the detection of antigens on subpopulations of epidermal cells and these cells have been sorted for further experimentation [8,9]. These studies have focused on the purification of Langerhans cell populations and have further emphasized the relevance and additional advantages of FACS analysis for use in detecting skin-specific antigens.

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